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OXIDATIVE STRESS AND ANTIOXIDANT ACTIVITIES OF PARACHLORELLA KESSLERI AT DIFFERENT GROWTH PHASES

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Abstract: *Parachlorella kessleri* is a potential source of natural antioxidants. However, limited research has been undertaken on *P. kessleri*'s antioxidants, especially during its cultivation period. This study aims to compare the oxidative stress and antioxidant activities in *P. kessleri* throughout different growth phases. It was grown in F/2 medium supplemented in natural seawater (30 ppt) under continuous light at 24±2°C for 24 days. The oxidative stress responses (hydrogen peroxide, H_2O_2 and malondialdehyde, MDA) and antioxidant assays (superoxide dismutase, SOD; catalase, CAT; ascorbate peroxidase, APX and glutathione reductase, GR) were assessed at five distinct phases; lag (day 1), exponential (day 4), early stationary (day 8), mid stationary (day 16), and death (day 24). Significant differences were observed for H_2O_2 during the exponential to stationary phases, but MDA was highest at the death phase. SOD, CAT, and APX activities were enhanced during the lag, exponential, and early stationary phases, respectively. GR reached the maximum activity during the exponential and early stationary phases. The results revealed that these oxidative stress biomarkers and antioxidant activities responded differently throughout growth phases, substantially at the exponential and early stationary phases. These findings are essential in selecting the best growth phase for further enhancements of *P. kessleri*'s antioxidants.

Keywords: *Parachlorella kessleri*, oxidative stress, antioxidants, growth phases.

Introduction

As aquatic relatives of plants, microalgae flourish in aerated, liquid environments with ample illumination, carbon dioxide, and other nutrients without the need for productive land and irrigation (Ezzeroual & El Kadmiri, 2021; Thanigaivel *et al*., 2022). They are rich in bioactive natural compounds like antioxidants, proteins, polyunsaturated fatty acids, and vitamins and have a number of benefits (Mobin *et al*., 2019; Gauthier *et al*., 2020). Currently, microalgae have been at the centre of research for various purposes such as aquaculture, biodiesel, cosmeceuticals, food, pharmaceuticals, and nutraceuticals (Hemaiswarya *et al*., 2011; Takeshita *et al*., 2018; Jiménez-Bambague *et al*., 2021; Qi *et al*., 2023). The worldwide market value of microalgae-based bioproducts is predicted to reach more than 53.43 billion USD by 2026 (Mehariya *et al*., 2021). However, the production of valuable bioactive compounds could be altered by the growth phases and culture conditions within the same strain of species (León-Vaz *et al*., 2023). Therefore, various studies have been performed to promote growth and alter the metabolic compounds of numerous microalgae strains to make bioproduct production, commercially viable (Bauer *et al*., 2017).

The Chlorophyta *Parachlorella kessleri* (Figure 1), also known as *Chlorella kessleri* is one of the best candidates for natural antioxidants because it produced the highest astaxanthin among nine microalgae studied by Soares *et al*. (2019). It is a coccoidal-shaped green microalga with 2.5 to 8.9 µm of cell size

(Juárez *et al*., 2011; Takeshita *et al*., 2018; Kević *et al*., 2019). As *P. kessleri* accumulates high levels of starch, lipid, protein, vitamins, and antioxidants, many researchers have studied its physiology and biochemical composition (Hemaiswarya *et al*., 2011; Piasecka *et al*., 2019; Liu *et al*., 2022). Moreover, *P. kessleri* has been reported to be a good lipid producer and is recognised to be safe for human consumption by the Food and Drug Administration (FDA) (Bauer *et al*., 2017; Manoyan *et al*., 2022). For the aquaculture industry, microalgae ought to meet various criteria, such as easy cultivation, absence of toxicity, high nutritive value, and should be easily digested (Hemaiswarya *et al*., 2011). In addition, *P. kessleri* is flexible and able to grow in photoautotrophic, heterotrophic, and mixotrophic cultures, which makes it a good candidate for worldwide commercialisation (Deng *et al*., 2019). In this study, *P. kessleri* was collected from Setiu Wetland, Terengganu, Malaysia and identified by using the molecular DNA identification by Tiong *et al*. (2020), which has the potential as a heat shock protein 70 enhancer in the brine shrimp *Artemia franciscana.* This finding leads to further questions on its oxidative stress and antioxidant activities during the cultivation period.

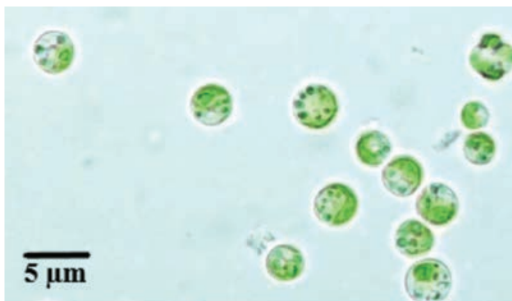

Figure 1: Microscopic observation of *P. kessleri* cells (Kević *et al*., 2019)

During the growth stage, microalgae pass through four distinct developmental phases, which are lag (almost no growth), exponential (linear increase in cell growth), stationary (constant in cell growth), and death (decline of cell growth) (Figure 2) (Price & Farag, 2013; Zabochnicka, 2022). They produce reactive oxygen species (ROS) comprising of free radicals with one or more unpaired electrons (superoxide anions and hydroxyl radicals) and non-radicals (hydrogen peroxide and singlet oxygen) during normal cellular metabolisms from photosynthesis, intracellular metabolic pathways, enzymatic reactions, or photorespiration (Chokshi *et al*., 2017; Roy *et al*., 2021; Kessler *et al*., 2022). ROS at optimal levels may have an effect on metabolisms and other signals, thus giving rise to biochemical accumulation (Zhao *et al*., 2022). However, a higher accumulation of ROS will initiate oxidative stress conditions causing injury to cellular macromolecules like proteins, lipids, and DNA, thus inhibiting cell growth and development (Aklakur, 2018; Roy *et al*., 2021; Zhao *et al*., 2022). A wide range of antioxidant defence mechanisms is produced in microalgae to confront excessive ROS and oxidative stress. They consist of enzymes such as superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase, as well as non-enzyme molecules like ascorbic acid, carotenoids, tocopherols, and glutathione (Smerilli *et al*., 2019). However, the oxidative stress and antioxidant defence mechanisms of microalgae may be altered over their growth phases (Canelli *et al*., 2020).

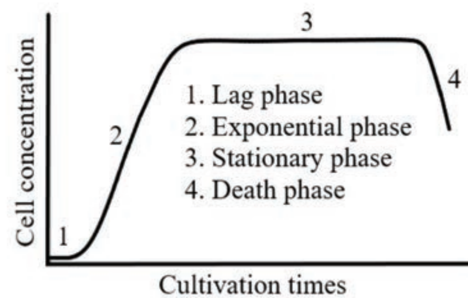

Figure 2: Growth phases of microalgae (Price and Farag, 2013)

Exponential and stationary phases are two common harvesting phases in various microalgal antioxidant studies (Canelli *et al*., 2020; González-Vega *et al*., 2021; Silva *et al*., 2021). However, the data only represents those particular phases, without considering variations

in antioxidant composition throughout the cultivation period. Furthermore, studies related to the antioxidant content of *P. kessleri* throughout the cultivation period are scarce. Therefore, this study was carried out to determine the oxidative stress biomarkers (hydrogen peroxide and lipid peroxidation) and antioxidant activities (superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase) of *P. kessleri* throughout its growth and developmental phases (lag, exponential, early stationary, mid stationary, and death). Based on previous studies, it is postulated that *P. kessleri* will exhibit various contents of oxidative stress responses and antioxidant activities at different phases of growth. This study is important for a better understanding on how to select the best phase for future optimization and enhancement of antioxidants in *P. kessleri*.

Materials and Methods

Cultivation of Parachlorella kessleri

Parachlorella kessleri was cultured and sustained in the Science and Technology Research Partnership for Sustainable Development (SATREPS) Laboratory, Institute of Marine Biotechnology, Universiti Malaysia Terengganu, 21030, Kuala Nerus, Terengganu, Malaysia. For this experiment, the culture was grown in 500 mL conical flasks filled with 400 mL of 30 ppt filter-sterilized seawater enriched with F/2 medium (Guillard & Ryther, 1962; Guillard, 1975). The culture was maintained under 24±2°C at 2,000 lux of LED light. The experimental cultures were performed in three batches with three replicates in each batch.

Harvesting of Cells

P. kessleri culture was harvested at five different phases, consisting of lag (day 1), exponential (day 4), early stationary (day 8), mid stationary (day 16), and death (day 24) which referred to our *P. kessleri* calibration growth curve. The liquid culture was harvested in 50 mL sterile tubes by centrifugation (Beckman Coulter Allegra X-30R Centrifuge, Germany) at 10,000 rpm for 10 minutes at 4°C. The cells were rinsed twice with distilled water to remove the dissolved salts in the medium. Fresh sample paste was used for oxidative stress and antioxidant analyses.

Analysis of Oxidative Stress Biomarkers

Two oxidative stress biomarkers, namely hydrogen peroxide (H_2O_2) concentration and lipid peroxidation by the evaluation of malondialdehyde (MDA) concentration in *P. kessleri* were performed following the method of Norhayati *et al*. (2017).

For the sample extraction, 0.05 g of fresh microalga sample was homogenized in an ultrasonic bath (Fisherbrand™ Elmasonic S 60H, Germany) for 10 minutes at 0 to 4°C in the presence of 1.0 mL of 0.1% trichloroacetic acid (TCA). The mixture was centrifuged (Beckman Coulter Microfuge 20R, Germany) at 10,000 rpm for 10 minutes at 4°C to collect the supernatant. To evaluate the H_2O_2 content, 50 µL of supernatant was transferred to a 96-well plate, with 50 µL of 10 mM potassium phosphate buffer (pH 7.0). The reaction was initiated by adding 100 µL of 1.0 M freshly prepared potassium iodide. The plate was incubated in a microplate reader (Thermo Fisher Scientific Varioskan™ LUX, Finland) for 10 minutes. Then, the absorbance was measured at 390 nm and the H_2O_2 concentration (mM/g fresh weight) in *P. kessleri* was calculated based on the calibration curve ($y = 1.1024x + 0.0636$) constructed using 0 to 0.16 mM H_2O_2.

Lipid peroxidation assay in *P. kessleri* was estimated based on the formation of MDA. A total of 0.75 mL of supernatant was diluted to a total volume of 3.0 mL with TBA reagent (prepared by the dilution of 0.5 g TBA in 100 mL of 20% TCA) and subsequently heated for 30 minutes at 95°C. Immediately, the mixture was cooled on ice for 15 minutes before 10 minutes of centrifugation (Beckman Coulter Microfuge 20R, Germany) at 10,000 rpm. The supernatant (200 µL) was transferred to a 96-well plate, and the absorbance was monitored at two wavelengths, 532 and 600 nm using a microplate reader (Thermo Fisher Scientific

Varioskan™ LUX, Finland). The MDA content (µmole MDA/g fresh weight) in *P. kessleri* was determined by subtracting absorbance values between both wavelengths divided by its extinction coefficient (155 mM/cm).

Analysis of Antioxidant Activities

For antioxidant activities of *P. kessleri* throughout the growth phases, four assays have been conducted, which were superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR). All antioxidant assays were expressed as U/mg protein.

SOD activity was conducted by the nitro blue tetrazolium (NBT) method adapted from Beauchamp and Fridovich (1971) and Price *et al*. (1994). One unit (U) of SOD activity was defined as the amount of enzyme that inhibited the rate of NBT reduction by 50percent at 560 nm per milligram microalgae protein. A total of 0.05 g of fresh sample was homogenized with 1.0 mL of 100 mM phosphate buffer (pH 7.0) for 10 minutes at 0 to 4°C using an ultrasonic bath (Fisherbrand™ Elmasonic S 60H, Germany). The mixture was centrifuged (Beckman Coulter Microfuge 20R, Germany) at 10,000 rpm for 10 minutes at 4°C. A total of 5 µL of supernatant was added to a 96-well plate filled with 195 µL of SOD reaction mixture containing 50 mM phosphate buffer (pH 7.8), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM NBT, 0.048 mM xanthine oxidase, and 0.05 mM xanthine. The SOD activity of *P. kessleri* was measured using a microplate reader (Thermo Fisher Scientific Varioskan™ LUX, Finland) at 560 nm.

CAT activity was determined according to the method of Claiborne (1985). One U of CAT activity was described as the degradation of 1 µmol H_2O_2 per minute per milligram microalgae protein. Fresh sample (0.05 g) was homogenized for 10 minutes at 0 to 4°C with an ultrasonic bath (Fisherbrand™ Elmasonic S 60H, Germany) in the presence of 1.0 mL of 50 mM phosphate buffer (pH 7.4). The mixture was centrifuged (Beckman Coulter Microfuge 20R, Germany) at 10,000 rpm for 10 minutes at 4°C for supernatant accumulation. The CAT reaction mixture contained 3.0 mL of 19 mM H_2O_2 in 50 mM phosphate buffer (pH 7.0) and 100 mL of supernatant was added into quartz cuvette. CAT activity was measured by the rate of the changes in absorbance at 240 nm over 2 minutes in a spectrophotometer (Shimadzu UV-1601, Japan).

APX activity was analysed based on the method by Nakano and Asada (1981) and Murshed *et al*. (2008). One Unit of APX activity was explained as the oxidation of 1 µmol ascorbate per minute per milligram microalgae protein. A total of 0.05 g of fresh sample was homogenized for 10 minutes at 0 to 4°C with an ultrasonic bath (Fisherbrand™ Elmasonic S 60H, Germany) in the presence of 1.0 mL of 100 mM phosphate buffer (pH 7.0) containing 1 mM L-ascorbic acid. The mixture was centrifuged (Beckman Coulter Microfuge 20R, Germany) at 10,000 rpm for 10 minutes at 4°C. A total of 10 µL of supernatant was added to a 96-well plate filled with 185 µL of APX reaction mixture consist of 50 mM potassium phosphate (pH 7.4) containing 0.025 mM L-ascorbic acid and 5 µL of 200 mM H_2O_2. APX activity was measured by the rate of the changes in absorbance at 290 nm over 5 minutes in a microplate reader (Thermo Fisher Scientific Varioskan™ LUX, Finland).

GR activity was performed following the method of Carlberg and Mannervik (1985). One U of GR activity was defined as the degradation of 1 mmol nicotinamide adenine dinucleotide phosphate (NADPH) per minute per milligram microalgae protein. Fresh sample (0.05 g) was homogenized with 1.0 mL of 50 mM phosphate buffer (pH 7.4) for 10 minutes at 0 to 4°C using an ultrasonic bath (Fisherbrand™ Elmasonic S 60H, Germany). The mixture was centrifuged (Beckman Coulter Microfuge 20R, Germany) at 10,000 rpm for 10 minutes at 4°C. The supernatant (50 µL) was added to a 96-well plate filled with 150 µL of GR reaction mixture containing 100 mM phosphate buffer (pH 7.0), 1.0 mM oxidized glutathione, 1.0 mM EDTA, and 0.1 mM NADPH. GR activity was measured

by the rate of the changes in absorbance at 340 nm over 3 minutes in a microplate reader (Thermo Fisher Scientific Varioskan™ LUX, Finland).

In each antioxidant activities assay, the protein concentrations of *P. kessleri* were measured following the method of Bradford (1976) with an adaptation to microplate reader usage. About 50 µL of extracted supernatant was transferred to a 96-well plate, with 150 µL of Bradford's reagent (prepared by the dilution of 0.1 g of Coomassie Brilliant Blue G-250 in 50 mL of 95percent of ethanol, 100 mL of concentrated phosphoric acid, and 850 mL of distilled water). The 96-well plate was incubated in a microplate reader (Thermo Fisher Scientific Varioskan™ LUX, Finland) for 10 minutes. Then, the absorbance was measured at 595 nm (A_{595}) and the protein concentration (mg protein) in *P. kessleri* was calculated based on the calibration curve (y = 0.00349x - 0.00637) constructed using 0 to 0.1 mg/mL of Bovine Serum Albumin.

Statistical Analysis

All collected data in this study were presented as Mean ± Standard deviation (SD). The data was analysed with one-way analysis of variance (ANOVA) using the Statistical Package for Social Science software (SPSS) 20 package for Windows to see the impact of the growth phases on oxidative stress and antioxidant activities of *P. kessleri*. Differences in data were considered significant at the $p<0.05$ level, and the data was further differentiated by post-Hoc comparisons using Tukey's test.

Results and Discussion

The oxidative stress biomarkers (H_2O_2 and MDA concentrations) and antioxidant activities (SOD, CAT, APX, and GR) of *P. kessleri* throughout its growth phases (lag, exponential, early stationary, mid stationary, and death phases) are further illustrated and discussed in this section.

Oxidative Stress Biomarkers of P. kessleri throughout Different Growth Phases

Oxidative stress occurs when excess ROS production is over the capacity of antioxidants causing localized oxidative damage by disrupting cell functions through lipid peroxidation, oxidizing proteins, and attacking nucleic acids (Rezayian *et al*., 2019). H_2O_2, one type of ROS, is a by-product of cellular mechanisms like photosynthesis and photorespiration (Battah *et al*., 2015; Bouzidi *et al*., 2022). Within the cells, the H_2O_2 has a long lifespan and functions as a cellular signalling cascade in normal concentration but becomes a toxic substance when present at high concentration, thus leading to oxidative stress (Battah *et al*., 2015; Roach *et al*., 2015; Szabó *et al*., 2020).

Figure 3 showed the oxidative stress biomarkers of *P. kessleri* at distinct growth phases. The H_2O_2 concentration of *P. kessleri* was significantly higher ($p<0.05$) at the exponential and early stationary phases, followed by a steep drop afterwards (Figure 3a). In contrast, Çakmak *et al*. (2015) reported that *Chlamydomonas reinhardtii* maintained H_2O_2 levels from the lag to early stationary phases. Interestingly, the H_2O_2 concentration in *Nannochloropsis oceanica* was noticed higher at the death phase under control conditions (Reshma & Arumugam, 2022).

For lipid peroxidation, an increment pattern of MDA content from the beginning to the early stationary phase was observed in *P. kessleri* in Figure 3b as reported in wild-type *C. reinhardtii* by Tran *et al*. (2019). *P. kessleri* exhibited the first rise of MDA concentration at the early stationary phase before a drastic second rise was observed at the death phase as observed in *Acutodesmus obliquus* (Piotrowska-Niczyporuk *et al*., 2018). On the other hand, MDA concentration was achieved higher at the lag phase in *Chlorella vulgaris* (Kong *et al*., 2022), whereas *N. oceanica* obtained the highest MDA at the lag and early stationary phases (Reshma & Arumugam, 2022).

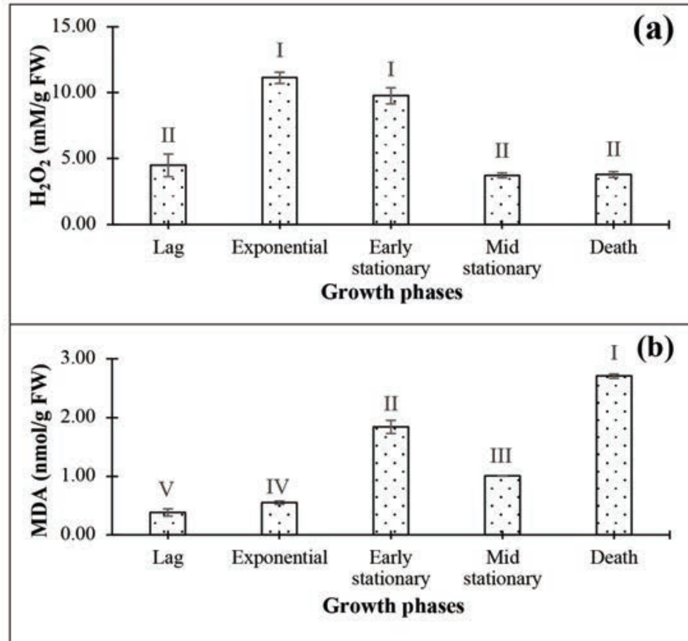

Figure 3: Oxidative stress (a) H_2O_2 and (b) MDA concentrations of *P. kessleri* at different growth phases. Data shown are mean ± SD (n=3). Different superscripts on error bars represented significantly different at $p<0.05$

As one of the most stable ROS, the amount of H_2O_2 produced is associated with the size of oxidative changes and the ability of the cells to recover from stress, hence returning to original conditions (Roach *et al*., 2015; Norhayati *et al*., 2017; Szabó *et al*., 2020). Based on Figure 3a, the increase of H_2O_2 during the exponential phase indicates its function in active cell growth and division as a cellular redox-controlled that needs regulation of H_2O_2 which is similar as that reported in *A. obliquus* (Roach *et al*., 2015; Krishnan *et al*., 2015; Piotrowska-Niczyporuk *et al*., 2018). However, when *P. kessleri* reached the early stationary phase, higher H_2O_2 concentration might be attributed to the deficiency of nutrients in the medium, which may promote an imbalance in the cellular redox pathways by the generation of hydroxyl (OH) radicals, causing oxidative stress (Krishnan *et al*., 2015; Roach *et al*., 2015). The drop in H_2O_2 level when *P. kessleri* reached the last two phases is related to the decline of photosynthetic pigments because H_2O_2 is primarily generated in chloroplasts (Javier *et al*., 2018; Wang *et al*., 2018; Szabó *et al*., 2020).

Lipid peroxidation is one of the most studied topics related to oxidative stress detection because ROS can alter the structural and functional membrane, resulting in membrane lipid damage (Danouche *et al*., 2020). In this study, the amount of lipid peroxidation in *P. kessleri* was assessed by the quantification of the MDA concentration. MDA content levelled up from lag to the early stationary phases suggesting the elevation of ROS-forming lipid peroxides initiates the oxidization of polar lipids in the cell membrane that engage in lipid bilayer formation and protective barrier for cells (Hasim & Yusuf, 2017; Kong *et al*., 2022). However, MDA went down during the mid stationary phase might be because of antioxidants' participation and improvement in scavenging ROS accumulation (Chokshi *et al*., 2017; Kong *et al*., 2022). The highest MDA observed in the death phase which might be due to the lipid membrane being extremely damaged when the excessive ROS got rid of the hydrogen from the unsaturated chain of fatty acids into cytotoxic molecules like MDA, leading to cells inhibition (Rezayian *et al*., 2019; Reshma & Arumugam, 2022).

Antioxidant Activities of P. kessleri throughout Different Growth Phases

Stress-induced ROS formation is confronted by a wide range of antioxidants like SOD, CAT, APX, and GR (Chokshi *et al*., 2017). In Figure 4, the antioxidant activities of *P. kessleri* at five distinct growth phases were shown. SOD activity exhibited a descending pattern from the beginning to the early stationary phases. Our result was in accordance with the finding by Kong *et al*. (2022) where *C. vulgaris* produced higher SOD at the lag phase. In contrast, SOD was observed higher during the exponential phase in *Isochrysis galbana* (Su *et al*., 2017) and *Microcystis aeruginosa* (Jiao *et al*., 2022), as well as at the early stationary phase in *C. reinhardtii* (Çakmak *et al*., 2015).

When *P. kessleri* passed through the different growth phases, CAT was notably elevated at the exponential, whereas APX was remarkably boosted during the early stationary phase. Interestingly, *P. kessleri* exhibited higher GR activity at both the exponential and early stationary phases. The results of CAT, APX, and GR of *P. kessleri* differed from the findings by Çakmak *et al*. (2015) as *C. reinhardtii* exhibited constant activities of CAT, APX, and GR from the lag to early stationary phases. On the contrary, the highest CAT was observed at the lag phase in *C. vulgaris* (Kong *et al*., 2022), but remained stable from the lag to the exponential phase in *M. aeruginosa* (Jiao *et al*., 2022).

As photosynthetic organisms, enzyme antioxidants serve as a part of the important ROS scavenging mechanisms under oxidative stress circumstances. SOD is a prominent antioxidant enzyme found in all subcellular components of photosynthetic creatures that are vulnerable to ROS-mediated cellular oxidative stress (Danouche *et al*., 2020). During the cultivation period, *P. kessleri* exhibited the highest SOD activity at the lag phase, indicating its function as a first line defence against ROS toxicity by stimulating the rapid formation of H_2O_2 and oxygen molecules from the dismutation of superoxide radicals (Rezayian *et al*., 2019). During the lag phase, *P. kessleri* might be stressed because of adaptation to new surroundings such as medium, light, and pH (Krishnan *et al*., 2015). SOD activity went down from the lag to early stationary phases and remained at a relatively low point during the three last phases, suggesting that the generation of superoxide radicals was significantly reduced at the beginning of the growth phase (Wang *et al*., 2018).

CAT and APX are tetrameric and heme-containing enzyme antioxidants that participate in H_2O_2 removal to prevent the emergence of the highly reactive OH radicals (Randhawa *et al*., 2012; Ugya *et al*., 2020; Jiao *et al*., 2022). Therefore, the OH radicals ought to be restrained because they cause radical chain reactions like double bond addition and hydrogen abstraction, leading to oxidative damage of cells membrane, chloroplast, and DNA (Randhawa *et al*., 2012; Danouche *et al*., 2022). In this study, *P. kessleri* revealed that CAT and APX were higher at different growth phases. CAT was first generated in a higher amount during the exponential phase might be because it does not require cellular reducing equivalents to catalyse excessive H_2O_2 (Bouzidi *et al*., 2022). Furthermore, CAT engages in protecting unsaturated fatty acids from being oxidized in the cell membrane (Esperanza *et al*., 2017). In contrast, the highest APX activity was observed later during the early stationary phase potentially because APX needs ascorbate as its specific electron donor to reduce H_2O_2 into H_2O (Rezayian *et al*., 2019).

Apart from SOD, CAT, and APX, a NADPH-dependent enzyme known as GR is also important in sustaining redox equilibrium in the cellular environment by utilizing NADPH to reduce glutathione disulphide (GSSG) to glutathione (GSH), hence, retaining a high cellular GSH/GSSG proportion in the cell cytoplasm (Çakmak *et al*., 2015; Sun *et al*., 2018; Danouche *et al*., 2020). GR activity in *P. kessleri* was induced during exponential and early stationary phases might be associated with the capacity of the cells to quench high concentrations of ROS (Sun *et al*., 2018). Our results suggest that the rising of GR might be coupled with the rising in CAT during the

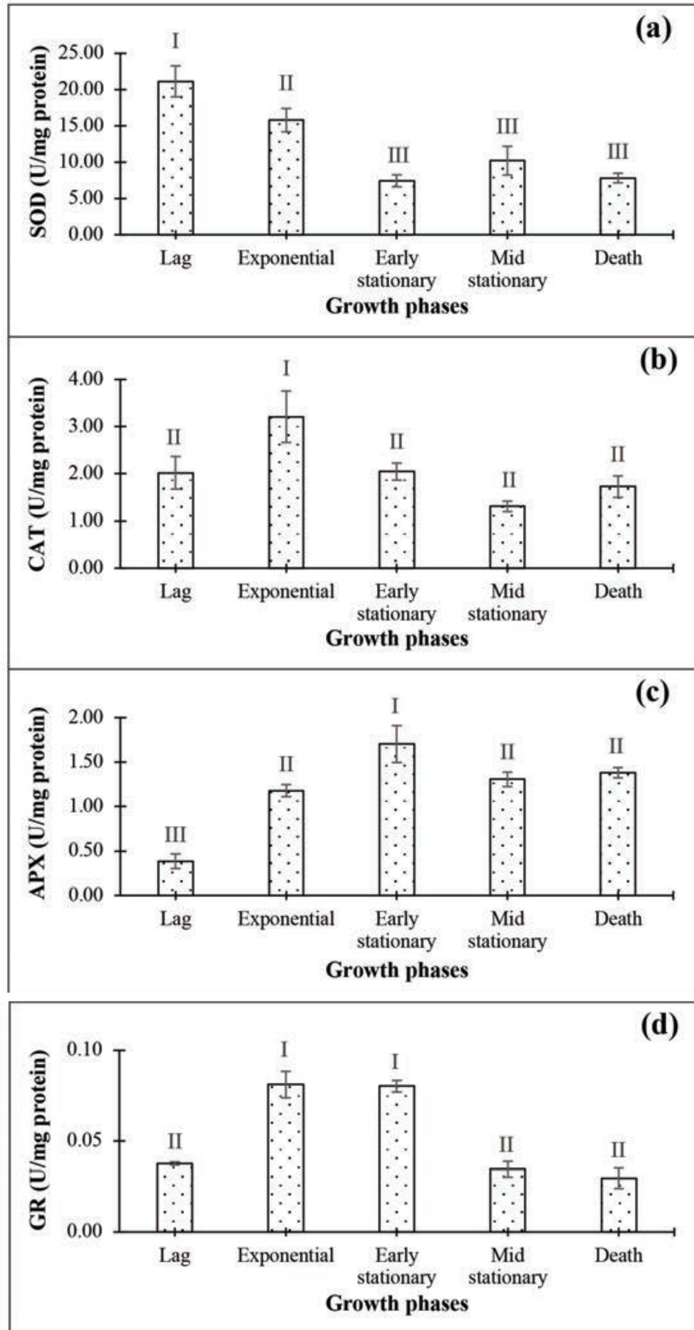

Figure 4: Antioxidant activities (a) SOD, (b) CAT, (c) APX, and (d) GR of *P. kessleri* at different growth phases. Data shown are mean ± SD (n=3). Different superscripts on error bars represented significantly different at $p<0.05$

exponential phase and APX during the early stationary phase. This synergic association of enzyme antioxidants is possibly related to the detoxification of H_2O_2 produced by the Mehler reaction in chloroplasts (Sharma *et al*., 2012; Szabó *et al*., 2020). The relation between GR and APX was supported in *Phaeodactylum tricornutum* because both antioxidants are involved in the Halliwell-Asada cycle, where GSH and NAPDH are utilized to revive the reduced form of ascorbate accumulation (Morelli *et al*., 2012; Danouche *et al*., 2022).

The reduction of all enzymatic antioxidants (SOD, CAT, APX, and GR) during the mid stationary and death phases may be because *P. kessleri* coincides with deprivation of essential nutrients in the cultivation medium (Javier *et al*., 2018). A previous study by Roy *et al*. (2021) spotted that the depletion of nitrogen diminished chlorophyll content as well as the synthesis of photosynthetic proteins in *Dunaliella salina*. In addition, the reduction of chlorophyll content has been reported to destruct peroxide of the thylakoid membranes in *P. kessleri* (Jiménez-Bambague *et al*., 2021). *P. kessleri* might have encountered a decline in photosynthetic pigments and deterioration of thylakoid membranes at the last two growth phases that lessened the photosynthetic rates and CO_2 fixation, thus exposing *P. kessleri* cells to oxidative stress (Javier *et al*., 2018).

Based on the results, the variations in the formation of oxidative stress biomarkers and antioxidants activities in *P. kessleri* throughout the growth phases might be influenced by several factors such as biological conditions of the microalgae like cell size, cell division, and cell density, as well as cultivation conditions like nutrient, carbon, and light availability (Krishnan *et al*., 2015; Ismaiel *et al*., 2016; Ugya *et al*., 2020). An increase in cell size, cell division, and cell density during the exponential phase leads to active metabolisms, thus accumulating higher ROS in the cells as a metabolic by-product (Ugya *et al*., 2020). When the cells reach the maximum density during the stationary phase, the cells will compete for the availability of nutrients, carbon, and light, which will initiate cell autolysis and self-degradation (Nasir *et al*., 2015; Ismaiel *et al*., 2016). Therefore, oxidative stress will function as an indicator of stress levels, whereas antioxidants serve as the defence mechanisms for the microalga cells' survival (Roy *et al*., 2021).

Conclusion

The above results indicated that the H_2O_2 concentration of *P. kessleri* was higher during the exponential and early stationary phases. In contrast, MDA levels were significantly higher during the death phase, suggesting major oxidative damage in lipid membranes. In antioxidant activities data obtained, SOD activity was levelled up during the lag phase, indicating its function as a first line defence in antioxidant mechanisms. Intensified production of CAT and APX enzymes was observed at the exponential and early stationary phases, accordingly. Remarkably, *P. kessleri* exhibited higher GR activity at both exponential and early stationary phases. It is suggested that the screening of antioxidant activities throughout distinct growth phases is important to select the optimum phase for further studies. In addition, further research on the manipulation of *P. kessleri*'s growth conditions should be conducted to enhance the production of antioxidant contents at the exponential or early stationary phase prior to commercialisation purposes.

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